APIGENIN AND NARINGENIN INCREASE APOPTOSIS AND DECREASE PROLIFERATION VIA TRANSCRIPTIONAL REGULATION

A Thesis

by

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ABSTRACT

Previous studies have shown that apigenin and naringenin (flavonoids) suppress colon carcinogenesis by inducing apoptosis and suppressing proliferation in rats. The goal of this thesis was to test the hypothesis that apigenin and naringenin affect colonocyte proliferation and apoptosis by regulating expression of genes involved in microbial recognition (TLR-2, TLR-4), short chain fatty acid transport (MCT-1), cell cycle (p21), and apoptosis (Bax, Bcl-2, Fas, Noxa) as well as to determine if the mechanism of action was p53 dependent. Scraped mucosa was obtained from rats which received diets (0.02% naringenin, 0.1% apigenin, or basal) for 10 wks and were treated with AOM. YAMC and mp53 YAMC cells were treated with apigenin (0.1, 1, and 10 μM), naringenin (0.1, 1, 10, 25, and 50 μM), or estradiol (1 nM, positive control) for 96 h at non-permissive conditions. *In vivo*, apigenin suppressed MCT-1 ($p<0.03$), Bax ($p=0.05$), and Fas ($p<0.05$) expression compared to the control diet; and both flavonoids suppressed p21 ($p<0.02$) and TLR-4 ($p<0.01$) expression. Diet did not affect expression of Bcl-2 or TLR-2. 1 μM or greater apigenin or naringenin treatment exhibited dose-dependent decreases ($p<0.005$) in cell numbers compared to vehicle in YAMCs, while no differences were identified in the mp53 YAMCs except with the highest treatment concentrations ($p< 0.0001$). No differences in proliferation were observed with apigenin or naringenin in either cell line, except with 20 μM apigenin treatment ($p< 0.0001$). Apoptosis and gene expression data were inconclusive *in vitro* due to a lack of response in the positive control. Considering MCT-1 is a butyrate transporter and butyrate induces

colonocyte p21 expression, the suppression of p21 expression may be a MCT-1 mediated effect. Reduction of MCT-1, p21, and TLR-4 expression by apigenin and naringenin suggests that these flavonoids may be able to reduce colon carcinogenesis through their influence on expression of genes involved in multiple pathways. The dose-dependent reduction in cell number induced by apigenin and naringenin is in part p53-mediated; however, the reduction in mp53 YAMC cells resulting from the greatest concentrations suggests alternate pathways can be induced. These reductions in cell number were not related to changes in proliferation.

DEDICATION

For my mother Donna Allison,

who has taught me to work hard, and who has herself worked hard,

so that I may succeed in all goals I pursue.

Thank you.

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CHAPTER I

INTRODUCTION

Cancer is second only to heart disease as the leading cause of death in the United States, accounting for nearly 1 in every 4 deaths. Cancer will most likely affect all Americans in some way or another during their lifetimes. The lifetime risk of developing cancer is 1 in 2 for men and 1 in 3 for women (1). According to the American Cancer Society, in 2014 it is expected that 585,720 Americans will die of cancer (1). Specifically, colon cancer is the third most common in the number of cancer cases and in the number of cancer related deaths for both men and women in the US, with an estimated 96,830 new colon cancer cases and 50,310 colorectal cancer deaths for 2014 (1). Upon diagnosis, the 5 year relative survival rate for those diagnosed with colon cancer is 64% in the US (2). Beyond the personal impact that cancer will have on individuals in society, it is also a major public health issue in regard to the significant burden to society. The estimate for overall costs of cancer in 2009 [the last year that costs were calculated by the National Institutes of Health (NIH)] was $216.6 billion, $86.6 billion for direct medical costs and $130.0 billion for indirect mortality (cost of lost productivity due to death) (1).

Although the incidence of colon cancer is high, it is predicted that 70% of colorectal cancers are preventable by changes in lifestyle and eating habits (3). It has been reported that diets containing elevated levels of fruits and vegetables are associated with decreased risk of colon cancer (4-8). Bioactive compounds from fruits and

vegetables and their role in colon cancer has been a focus of many studies in recent years. In our laboratory, grapefruit was shown to reduce aberrant crypt foci, the early neoplastic lesions of colon cancer (9). Grapefruit also increased colonocyte apoptosis, an important mechanism involved in removing damaged cells and decreased colonocyte proliferation (9). Further work demonstrated that apigenin and naringenin, two bioactive compounds found in grapefruit, are able to suppress colon carcinogenesis (10). The protection may have resulted, in part, because of the increase in apoptosis found in colonocytes lining the luminal surface and the suppression of proliferation. However, the mechanisms underlying these effects were not identified in those experiments. The experiments described in this thesis have extended the examination of those observations to elucidate the mechanisms involved in upregulating apoptosis and downregulating proliferation in colonocytes.

CHAPTER II

LITERATURE REVIEW

Colon Cancer

Cancer is a group of diseases resulting from uncontrolled cell growth. According to the multi-stage carcinogenesis model, cancer begins when a cell is initiated by exposure to a carcinogen that causes a DNA adduct (11). DNA adducts can lead to a tumor promoting DNA mutation. The initiated cell can undergo subsequent additional transformations that enable it to start forming a preneoplastic lesion, which may become a cancer (11).

In order for an initiated cell to progress through these stages to cancer, certain functional changes must occur. These changes include insensitivity to anti-growth signals, self-sufficiency in growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, and activation of tissue invasion and metastasis (12). Differential expression of oncogenes and tumor suppressor genes is known to be involved in the functional changes that occur in cells and contribute to cancer progression. Oncogenes are genes that can lead to cancer as a result of genetic or epigenetic changes in either their coding region or regulatory sequences. They can cause cancer by increasing cell proliferation and survival and by causing cell dedifferentiation, where a differentiated cell reverts to an earlier developmental stage (13). Tumor suppressor genes are genes that inhibit cell proliferation and survival. Inactivation or mutation of tumor suppressor genes also can increase the risk of tumorigenesis (13). In

colon cancer, multiple functional changes must occur to transform a normal cell into a malignant cell. Changes in oncogenes and tumor suppressor genes help an initiated cell gain the functional changes required to progress to cancer and metastasis.

One change that is necessary for tumor growth is the evasion of apoptosis, or programmed cell death (11-12). Two distinct signaling pathways can initiate apoptosis, the intrinsic and extrinsic pathways; and, the tumor suppressor gene p53 is involved in both pathways. In response to DNA damage, aberrant growth signals, or oxidative stress, p53 is activated and can subsequently trigger a wide array of physiological responses, including induction of apoptosis, initiation of DNA repair, and initiation of cellular senescence (14-15). Approximately 50% of colorectal carcinomas are associated with point mutations in p53, which are also correlated to an increase in disease severity (16-18). Once p53 becomes activated, it can alter the expression of downstream apoptotic genes, including Bax and Noxa (upregulate apoptosis) and Bcl-2 (downregulates apoptosis). The extrinsic pathway involves death receptor proteins (such as Fas) found on the surface of the cell (19). These death receptors bind to their respective ligands, assembling a death-inducing signaling complex. Fas is a major death receptor expressed in the normal colonic crypt (20-21). Both the intrinsic and extrinsic pathways converge in the caspase cascades at caspase-3, -6, and -7, which are the executioner caspases. Although there is low frequency of apoptosis in the colon (less than one apoptotic cell per crypt column), it remains important in the case of cancer progression (22). When apoptosis is dysregulated, cells that have DNA damage can continue to survive, promoting carcinogenesis (12).

Another change that is important is the insensitivity to anti-growth signals. $p21^{WAF1/CIP-1}$ is a downstream target of the p53 protein. The p21 protein inhibits cyclin dependent kinases (CDKs), which have a direct function in the transition between cell cycle phases, thus leading to cellular arrest (23). After p53 is activated as a result of DNA damage, p21 is upregulated through the AMP-activated protein kinase (AMPK) pathway (24). AMPK is involved in cellular energy balance as well as in regulation of proliferation and growth through the inhibition of the mTOR pathway and activation of p21 and p53 (24). This activation will lead to a decrease in proliferation, allowing the cell to correct the damage. p21 is not involved directly in cellular repair but allows cellular arrest so that the damage is not copied. One study revealed that loss of p21 accelerated tumor growth in mice expressing a mutant form of p53 that cannot induce apoptosis but retains partial cell cycle regulation, revealing that p21 is a major factor of tumor protection by p53 (25). When cellular proliferation is dysregulated, mutations become fixed and can be replicated during cell divisions, leading to the promotion and progression of carcinogenesis.

Beyond the traditional functional changes seen in cancer, the intestines are unique because of the direct interaction they have with the microflora and their metabolites. The fermentation of complex carbohydrates, such as dietary fiber, produces butyrate, a short chain fatty acid, which can modulate colon carcinogenesis progression (26-27). There is evidence that butyrate is capable of regulating changes in cell cycle, differentiation, and cell apoptosis (28-29). Specifically, butyrate has been shown to restore p21 expression to control levels following repression by azoxymethane (AOM), a

colon specific carcinogen (30). A possible mechanism of this restoration is epigenetic changes. In human skin cancer cells, Nandakumar et al. demonstrated that p21 expression could be restored by suppressing DNA methylation and by decreasing histone deacetylase (HDAC) activity with treatment of green tea polyphenols (31). Additionally, there is evidence that combinations of DHA and butyrate promotes apoptosis in part by inducing methylation of the anti-apoptotic genes, including Bcl-2 promoter (32). Epigenetic changes may be related to the fact that butyrate is a direct energy source in healthy colonocytes but not in typical cancer cells. Because cancer cells exhibit the Warburg effect (a high rate of glycolysis and lactic acid fermentation), their preferred energy source is glucose instead of butyrate (33-34). As a consequence, cancerous colonocytes accumulate butyrate within the nucleus, where it acts as a HDAC inhibitor, causing epigenetic regulation of gene expression (26). Butyrate's potential to cause epigenetic changes make it especially important in colon cancer prevention.

Beyond their fermentation products, the microbial population within the colon can come into direct contact with the colon epithelium. Toll-like receptors (TLRs), found on colonocytes, allow the cell to recognize commensal- and pathogen-associated molecular patterns from the gut microbiota (35). Therefore, the cell surface TLRs play a role in the immune and barrier function of intestinal epithelial cells; for example TLR-4 is important for the inflammatory response during injury (activating NF-kB and inducing expression of IL-1, -6 and -8), while TLR-2 is important for mucosal integrity (36-37). TLR-4 and TLR-2 specifically play vital roles in the response to lipopolysaccharides (LPS, large glycoproteins found on the outer membrane of Gram-negative bacteria) (38).

TLR-4 triggers both the MyD88- and TRIF-dependent pathways, while TLR-2 primarily signals the MyD88-dependent pathway. The MyD88 pathway ultimately results in the activation of NF-κB, leading to the induction of inflammatory mediators (39). Furthermore, enhanced inflammation is known to cause promoting and initiating events in carcinogenesis (11). The activation of NF-κB and subsequent inflammatory response related to changes in TLR expression and activation can lead to the promotion of colon carcinogenesis (35).

Colon cancer can be influenced by many environmental factors. It is predicted that 70% of colorectal cancers are preventable by changes in lifestyle and eating habits (3). It has been demonstrated that various bioactive compounds in fruits and vegetables have the ability to suppress early neoplastic lesions of colon cancer (9-10, 40-41). A potential mechanism of action could be the ability of dietary components to influence the homeostasis of the colonic epithelium through altering signal transduction pathways and transcriptional regulation.

Apigenin and Naringenin

Polyphenols are bioactive phytochemicals that contain aromatic rings with reactive hydroxyl groups. Specifically, the polyphenols of interest, apigenin and naringenin, are flavonoids. There are seven subclasses of flavonoids; apigenin is part of the flavone subclass, while naringenin is a flavanone. Apigenin and naringenin are very similar to one another, possessing the same functional groups attached in the same places; however, they differ by one additional double bond found on the aromatic ring of

apigenin at the C2-C3 position. This double bond makes apigenin more planar than naringenin (Figure 1). Polyphenols typically have a low bioavailability, and therefore reach the colon, where they can interact with colonocytes and the colonic microbiota (42).

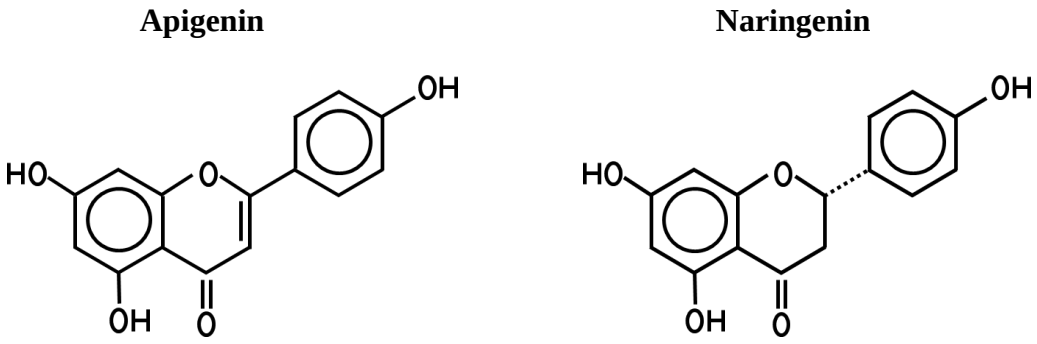

Figure 1. Structures of Apigenin and Naringenin. Apigenin and naringenin possess the same functional groups and differ in only one aromatic ring structure. Apigenin is a flavone, which has an additional double bond, compared to naringenin. Structures adapted from Liang et al. (43).

Besides grapefruit, parsley, celery and chamomile tea contain apigenin, while oranges and tomatoes contain naringenin. The daily intake of flavonoids has been estimated to be as high as 0.5-1.0 g in the American diet (value refers to total flavonoid intake) (44); however, other reports predict a lower actual daily intake in some cultures, ranging from 1-9 mg (South African diet) to 75-81 mg (Scandinavian diet) (values mostly refer to five flavonoids) (45).

In our laboratory, it has been demonstrated that apigenin and naringenin reduce the number of high multiplicity aberrant crypt foci (HMACF) in an AOM injected rat model (10). Aberrant crypt foci containing more than four crypts are referred to as

HMACF, and are correlated with subsequent formation of adenomas and adenocarcinomas (46-47). This protection may be due to the increased apoptosis on the luminal surface and decreased proliferation within the crypt. These phenotypic changes are likely downstream of alterations in transcription of genes in the p21 and caspase pathways as well as changes in the production of inflammatory mediators. Although the previous work in our lab identified no significant changes in the protein levels of Cox-2, an enzyme responsible for inflammatory mediators, or iNOS, a protein which can be induced to generate large amounts of nitric oxide involved in inhibiting the growth of invading microorganisms and neoplastic tissues (10), others have demonstrated changes in the expression of these genes with apigenin treatment (48-50).

The chemoprotective ability of apigenin and naringenin may be related to p53 expression and activity. McVean et al. demonstrated that apigenin treatment increased the half-life of p53 in mouse keratinocyte 308 cells, suggesting that apigenin may exert anti-tumorigenic activity by stimulating the p53 pathway (51). Moreover, Zheng et al. revealed that apigenin was able to induce apoptosis and G1 cell cycle arrest in cervical carcinoma cells, which was associated with increases in p53, p21, Bax, and Fas protein levels and reductions in Bcl-2 protein levels (52). Another study showed that apigenin caused induction of apoptosis in a human neuroblastoma cell line, but it did not change apoptosis in a corresponding mutant p53 expressing cell line (53). In that experiment, the increase in p53 protein levels upon apigenin treatment also led to an increase in protein expression of p21 and Bax in the human neuroblastoma cells but not in the mutated p53 cell line (53). In addition to alterations in the gene and protein expression of p53,

changes in gene expression of the downstream targets may also occur with apigenin or naringenin treatment. Park et al. showed that induction of apoptosis in human leukemia THP-1 cells treated with naringenin was in part due to downregulation of Bcl-2 and upregulation of Bax, resulting in activation of caspases (54). This induction of apoptosis was associated with the reduced activity of the PI3k/Akt pathway (54). Several other studies indicate that apigenin and naringenin work to activate the MAPK (ERK 1/2) pathway (55-57). Apigenin has also been demonstrated to inhibit the Noxa-induced phosphorylation of p38 MAPK, which is involved in the downregulation of the expression of matrix metalloproteinase-9 (MMP-9, can promote tumorigenesis through extracellular matrix remodeling and angiogenesis) (58). With these studies, there is marked evidence that apigenin and naringenin at least in part work through a p53-dependent pathway, affecting the activation of the apoptotic as well as the proliferation pathways.

In regards to cell cycle, Wang et al. reported a reversible induction of G2/M arrest by apigenin in three colon carcinoma cell lines (59). Apigenin induces G2/M cell cycle arrest, in part, due to increased p53 and p21 protein levels (60). Gupta et al. went further discovering an induction of p21 transcriptional upregulation in human prostate carcinoma cells (61). These studies indicate that cell cycle arrest and specifically increased expression of the p21 gene may explain, at least in part, apigenin's ability to reduce carcinogenesis.

Naringenin and apigenin may also impact apoptosis via changes in MCT-1 activity and butyrate availability. Naringenin was shown to decrease the activity but not

the expression of MCT-1 in Caco-2 (human colon cancer) cells, blocking uptake of benzoic acid by MCT-1 (62). Cellular accumulation of naringenin revealed that the decreased transport was not due to naringenin being used as a substrate of MCT-1 (62). Both compounds were also shown to reduce the uptake of γ-hydroxybutyrate in human breast cancer cells (MDA-MB231) (59). Moreover, increased lactate uptake in HT-29 (human colon cancer cells) with treatments of a flavone (the basic structure of apigenin) was associated with ROS production and ultimately apoptosis induction; but this apoptosis induction was significantly reduced by pCMBS, an MCT-1 inhibitor (63). These studies indicate that MCT-1 activity is altered in the presence of flavonoids and is related to apoptosis induction. In addition to MCT-1 activity, actual butyrate availability has been recognized to increase apoptosis. Butyrate has been shown to upregulate p21 expression (30). According to Fan et al., butyrate-induced apoptosis was increased by Fas and FasL and was blocked by inhibitors of FasL and caspases (21). With this in mind, MCT-1 expression and activity appear to be relevant to apigenin and naringenin's ability to reduce carcinogenesis, especially in regard to butyrate availability.

Beyond butyrate availability, the actual interaction of the microflora to the host can be impacted by the diet and can have an effect on disease states. TLRs allow communication between the colonic epithelium and the microbial population. One study showed that orange juice (a source of naringenin) prevented the elevated expression of IL-1β, TNFα, and TLR-4 and the induction of NF-κB binding, which was found in subjects given cream or glucose drinks in place of orange juice (except TLR-4, which was only elevated with cream drink consumption) (64). These same researchers also

showed that orange juice exerted a powerful inhibition of ROS generation (65). Moreover, luteolin, another flavonoid, suppressed the activation of TLR-4, blocking downstream target genes including TNFα and IL-6, in a mouse macrophage cell line (66-67). Additionally, Youn et al. discovered that a polyphenol component in green tea was able to suppress MyD88- and TRIF-dependent TLR signaling pathways in a murine monocytic cell line and a human embryonic kidney cell line (68). These studies provide further evidence that flavonoids can reduce inflammation, which is a promoter of carcinogenesis. Parkar et al. even discovered that naringenin was able to inhibit specific bacteria from adhering to Caco-2 cells (69). If naringenin and apigenin have the ability to alter the expression of TLRs or the gut microecology, then the inflammatory response as well as butyrate availability may be impacted, affecting downstream initiation and promotion events as well as apoptotic pathways.

Furthermore, apigenin and naringenin may have estrogenic effects. Estrogen has been shown to be protective against colon cancer (70-75). This is shown not only in the decreased incidence in pre-menopausal women compared to men but also in hormone replacement therapy (HRT) clinical studies (71, 73-75). Data from a collaborator's laboratory have demonstrated that estradiol (E_2) suppressed cell growth through induction of apoptosis in a non-transformed, non-malignant young adult mouse colonocyte primary cell line (YAMC bleo/neo) compared to a p53-null version of the same cells (YAMC mp53/neo) (76). Increased apoptotic activity along with a reduction in preneoplastic lesions was seen in ovariectomized wild-type mice treated with E_2 compared to wild-type control mice and the ERβ knockout mice receiving either

treatment (77). By binding with estrogen receptor beta (ERβ), E_2 activity is reported to protect against colon carcinogenesis via apoptosis induction and activation of p53 (tumor suppressor gene) (76-77). Yet HRT has risks related to cardiovascular disease and other estrogen sensitive cancers related to ERα (breast and ovarian) and may not be an acceptable option for men (78). Therefore, research has been targeted at the potential of phytoestrogens to mimic the effects of estrogen on colon cancer risk without the associated dangers. Phytoestrogens are plant derived non-steroid phenols with similar physiological functions as estrogen. A diet rich in soy, which contains phytoestrogens such as genistein, has been associated with chemo-protective benefits and reduced colon cancer risk (79). In fact, genistein treatment of non-transformed YAMCs led to an increase in p53 transcriptional activity (80). Naringenin has been identified as an ERα antagonist but is able to bind to ERβ, whereas apigenin shows affinity to both estrogen receptors (81); however, the role of apigenin and naringenin as phytoestrogens in colon cancer prevention is not yet clear. One study has shown that sorghum phenolic extracts (which contained apigenin) had estrogenic activity and also induced apoptosis in nonmalignant YAMCs (82). To determine if apigenin and naringenin may have estrogenic effects, E_2 was used as a positive control *in vitro* to induce apoptosis via upregulation of Bax and Noxa gene expression and downregulation of Bcl-2 gene expression

The literature indicates that apigenin and naringenin can modulate several of these cellular perturbations that increase cancer risk, including promotion of cell cycle arrest and apoptosis. Furthermore, naringenin and other flavonoids have been reported to

alter short chain fatty acid uptake by MCT-1 as well as inflammation regulated by TLR pathways. These changes appear to be related to transcriptional regulation by the flavonoids; therefore, this thesis has focused on understanding that regulation. Based on the mechanisms involved in the apoptotic and proliferation pathways within cancer development and research relating apigenin and naringenin to changes in gene expression or gene products within those pathways, several target genes were selected to measure expression levels. These include Bcl-2, Bax, Fas, and Noxa (involved in apoptosis regulation), p21 (cell cycle regulation), MCT-1 (short chain fatty acid transport), and TLR-2 and -4 (microbial recognition).

Hypothesis

We hypothesized that apigenin and naringenin lower the incidence of colonic preneoplastic lesions by regulating expression of genes involved in microbial recognition, short chain fatty acid transport, cell cycle, and apoptosis, especially those that may be p53 target genes.

Specifically, our aims were:

1. To determine the *in vivo* expression of Bcl-2, Bax, Fas, MCT-1, p21, TLR-2 and TLR-4 using scraped colon mucosa from rats fed apigenin, naringenin, or basal diets.

2. To determine the effects of apigenin and naringenin on conditionally immortalized (bleo/neo) and transformed (mp53/neo) YAMCs. Variables measured include:
    a. Cell number, proliferation, and apoptosis, and
    b. Gene expression of Bax, Bcl-2, p21, Noxa, and MCT-1.

CHAPTER III

MATERIALS AND METHODS

Experiment I—In Vivo *Gene Expression*

Scraped Mucosa from Rats. Colon tissue samples were acquired from a previous study done in our laboratory (10). Male Sprague Dawley rats (21-d) received either basal, apigenin (0.1%), or naringenin (0.02%) diets for 10 wks (10 rats/diet group). All rats were injected twice with 15 mg AOM per kg body weight, spaced 1 wk apart. The first injection was given after 3 wks of experimental diets. Rats were terminated 6 wks after the last AOM injection and colon tissue was collected (Figure 2). The tissue used in the present study was scraped mucosa that had been homogenized in lysis buffer solution, flash frozen, and stored at -80°C (Figure 3).

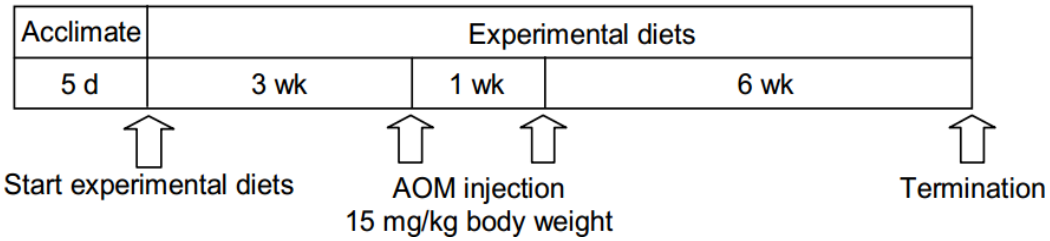

Figure 2. Timeline of Animal Study. Rats were acclimated for 5 d upon arrival and then stratified according to their body weights into 3 groups. Animals were given diets for 3 wks prior to the first AOM treatment. All rats were injected twice with AOM, spaced 1 wk apart. Rats were terminated 6 wks after the last AOM injection and colon tissues were collected. Figure from Leonardi (83).

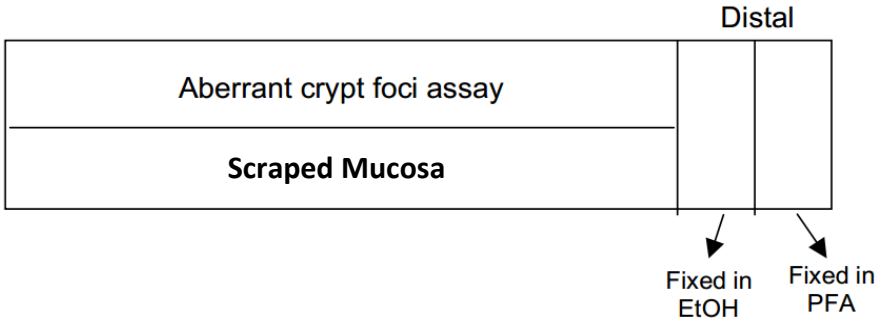

Figure 3. Schematic Diagram of Rat Colon Sample Collections. RNA was collected from the section labeled scraped mucosa. Abbreviations: EtOH, ethanol; PFA, 4% paraformaldehyde. Figure adapted from Leonardi (83).

RNA Isolation from Scraped Mucosa. RNA was isolated from scraped colon mucosa using the RNAqueous kit (Ambion), according to kit protocol and based on the procedures used by Monk et at. (84). After isolation, RNA was immediately treated with the DNA-free kit (Ambion), according to kit protocol, to remove all remaining DNA. The concentration and quantity of the isolated RNA was determined using a Nanodrop. Quality of the isolated RNA was measured on an Agilent 2100 Bioanalyzer. RNA integrity number (RIN) value of 10 indicates intact RNA, and a value of 8 and above is considered high quality RNA (85). We choose to use only RNA with a RIN value of 9 or higher for better and more consistent results in downstream experiments.

Gene Expression Using RNA Isolated from Scraped Mucosa. Prior to completing reverse transcription (RT) and gene expression, a trial was performed to determine what concentration of RNA in the RT reactions would be optimal for analysis.

It was determined that by using 150 ng of RNA the expression would be in the linear range of all target genes.

After assuring that the isolated RNA was intact, RT reactions to prepare cDNA from the isolated total RNA were performed according to the procedures of Fan et al. (86), using SuperScript III Reverse Transcriptase with the addition of random hexamers. Minus RT reactions (water was used in place of SuperScript III) were used as the negative control. The cDNA was aliquoted into microcentrifuge tubes to decrease the amount of thawing and refreezing, which can damage cDNA.

Expression of Bcl-2, Bax, Fas, p21, MCT-1, TLR-2, and TLR-4 was measured by real-time quantitative Reverse Transcription PCR (qRT-PCR) using TaqMan Gene Expression Master Mix and Taqman Primers (86). Each plate was set up with all samples plus two minus RT reactions in duplicate for the gene of interest. A positive control gene (18S) for each sample and a minus RT reaction for 18S were also included. The real-time qPCR was completed under standard conditions using the FAM detector on an Applied Biosystems 7900HT Fast Real-Time PCR System for 40 cycles.

Experiment II—In Vitro *Effects*

Cell Maintenance. Young Adult Mouse Colonocytes (YAMCs), bleo/neo (non-transformed) and mp53/neo (mutated p53 protein), were utilized for the *in vitro* experiments to assess whether apigenin or naringenin's effect on apoptosis and proliferation is p53-mediated. YAMCs were supplied by Dr. Clinton Allred (Department of Nutrition and Food Science, Texas A&M University, College Station, TX). Cells

were maintained as previously described by Weige et al. (76). Briefly, YAMCs were cultured in RPMI 1640 media (Sigma-Aldrich), containing 10% (v/v) fetal bovine serum (FBS; HyClone), 1xITS-A (BD Biosciences), and 2.5 µg/ml gentamycin (GIBCO), and were maintained on rat tail collagen type 1 (BD Biosciences) coated plates at the permissive conditions of 33°C and 5% CO_2 with 5 units interferon gamma (IFN-γ)/ml media (Roche) (76). Media was changed approximately three times per wk, and cells were split when they became nearly 100% confluent on the plate. YAMCs were only passaged for 60 d past the initial thaw date. All replicates of one type of experiment were conducted using cells from the same batch of thawed cells.

Apigenin and Naringenin Concentrations for Cell Culture. Based on the low bioavailability of apigenin and naringenin and the subsequent high concentration in the colon (42) and considering the percent of apigenin or naringenin in the rat diet, it was predicted that the concentrations of apigenin and naringenin within the rat colons in the *in vivo* study were 74.47 µM and 24.73 µM, respectively. This is the formula used for this calculation:

Colon [flavonoid] = % flavonoid in diet X wt. of food intake (g) X (molecular wt. of flavonoid)$^{-1}$ X 10^{-6} µM/M.

Based on the projected colon concentrations and other cell culture studies (52, 54, 61, 87-88), a preliminary cell growth study was conducted with 25, 50, 100, and 200 µM treatments of apigenin and naringenin. During this time, it was determined that treatment of 50 µM or higher apigenin and treatment of 200 µM naringenin to the non-

transformed YAMCs was toxic to the cells. At 25 µM, apigenin was shown to lower cell number to approximately 10-15% of the vehicle. Therefore lower concentrations were tested, and the decision was made to treat cells with 0.1, 1, and 10 µM apigenin and 0.1, 1, 10, 25, and 50 µM naringenin for the cell growth assay. Based on data in the cell growth assay, the concentrations of apigenin and naringenin that elicited reductions in cell number similar to estradiol were selected for proliferation, apoptosis, and *in vitro* gene expression analysis.

Cell Growth Assay. YAMC bleo/neo and mp53/neo cells were split into charcoal dextran stripped FBS media in the presence of IFN-γ. After 2 d, cells were collected and counted using a hemocytometer. Approximately 15,000 cells were seeded in each well on a collagenized 6-well plate. Cells were kept in permissive conditions, 33°C, for 24 h, allowing them to adhere to the plate overnight. Cells were treated with media containing apigenin or naringenin for 96 h and were maintained at non-permissive conditions, 37°C, without the presence of INF-γ and with media changed every 48 h (Figure 4). Treatments included apigenin (0.1, 1, and 10 µM), naringenin (0.1, 1, 10, 25, and 50 µM), estradiol (1 nM; positive control) and DMSO (vehicle; not exceeding 10% in the media). Because of the known effects seen in these cells on cell growth, apoptosis, and p53 target genes by estradiol treatment, estradiol was used as a positive control in our *in vitro* experiments (76). After 96 h of treatment, cells were collected and counted using a coulter counter (76). Three wells per treatment per experiment were used and five replicate experiments conducted.

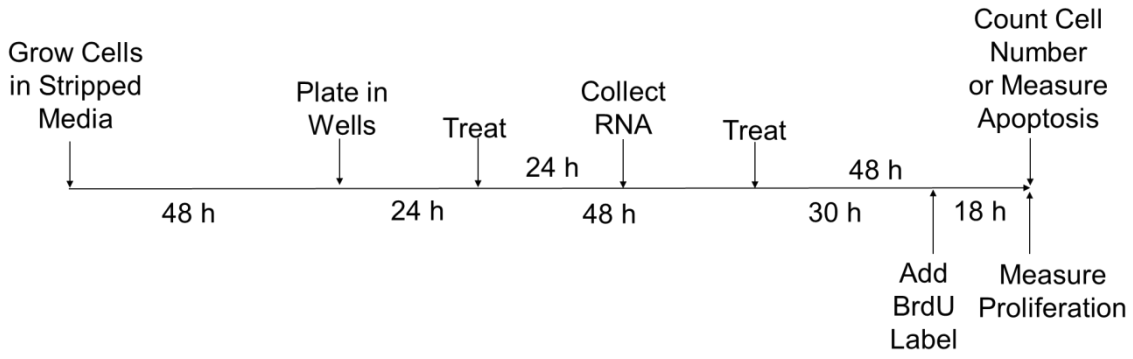

Figure 4. Timeline of Cell Culture Experiments. YAMCs were acclimated for 2 d in media stripped of hormones, including estrogen. The same number of cells was plated in each well. Cells were treated 24 h after plating and again 48 h post initial treatment. For proliferation experiments, BrdU was added 18 h prior to final assay. Cell number, proliferation, or apoptosis was measured at 96 h after initial treatment. For gene expression, RNA was collected after 24 h of treatment. Each run included 3 wells for each treatment group and at least 3 runs were conducted for each type of assay.

Proliferation. YAMC bleo/neo and mp53/neo cells were split into charcoal dextran stripped FBS media in the presence of IFN-γ. After 2 d, cells were collected and counted using a hemocytometer. Approximately 2,000 cells were seeded in each well on a collagenized 96-well plate. Cells were kept in permissive conditions for 24 h, allowing them to adhere to the plate overnight. Cells were treated for 96 h and media changed every 48 h (Figure 4). Treatments included 0.1 or 1 µM apigenin, 1 or 10 µM naringenin, 1 nM estradiol, and DMSO (vehicle). Estradiol was not shown to reduce proliferation in non-transformed YAMCs (80); therefore 20 µM apigenin was used as a positive control based on G1 cell cycle arrest and induction of p21 protein reported in human prostate carcinoma cells with this concentration of apigenin (61). After treatment, cells were maintained at non-permissive conditions, 39°C without the presence of INF-γ. At 78 h, Bromodeoxyuridine (BrdU), from the CalBiochem BrdU Cell Proliferation

Assay kit (Calboichem) was added; and cells were allowed to incubate for 18 h. BrdU, a synthetic analog of thymidine, can replace it during DNA synthesis and be measured to indicate cellular proliferation (80). BrdU was incorporated using the kit protocol and was based on the procedures used by Billimek (80), which includes measuring fluorescence on a TECAN Infinite M200 plate reader at wavelengths of 450 and 540 nm. Three wells per treatment per experiment were used and three replicate experiments conducted.

Apoptosis. YAMC bleo/neo and mp53/neo cells were seeded in the same manner as the cell number assay. Cells were maintained under non-permissive conditions, 39°C, in charcoal-dextran stripped FBS media. Treatments included 0.1 or 1 μM apigenin, 1 or 10 μM naringenin, 1 nM estradiol (positive control), and DMSO (vehicle). After 96 h of treatment, cells adherent to the plate and cells floating in the media were collected and lysed (Figure 4). Caspase activity was measured using EnzCheck Caspase-3 Assay Kit #2 (Molecular Probes), with various concentrations of Rhodamine 110 diluted in 1X reaction buffer used as standards and lysis binding solution used as a non-enzyme control (76). Fluorescence was measured on a TECAN Infinite M200 or SpectraMax Gemini EM plate reader (consistent between runs in each attempt) at wavelengths of 496 and 520 nm at 15 min intervals from 30 min up to 1 h. Three wells per treatment per experiment were used and at least three replicate experiments conducted.

Gene Expression. Approximately, 80,000 YAMC bleo/neo and mp53/neo cells were seeded in each well of a collagenized 6-well plate and maintained under non-

permissive conditions, 39°C, in charcoal dextran stripped FBS media. Cells were grown for 72 h and treated during the final 24 h (Figure 4). Treatments included 0.1 or 1 µM apigenin, 1 or 10 µM naringenin, 1 nM estradiol, and DMSO (vehicle). There were three wells per treatment and three replicate experiments conducted. RNA was isolated using the Direct-zol™ RNA MiniPrep kit (Zymo Research), according to kit protocol. RNA was in-column DNase I treated. Immediately after isolation, the concentration of the isolated RNA was determined using a Nanodrop. Quality of the isolated RNA was measured on an Agilent 2100 Bioanalyzer. We chose to use RNA with a RIN value of 9 or higher for better and more consistent results in downstream experiments, except for two samples that had RIN values of 8.7 and 8.9.

Prior to completing reverse transcription (RT) and gene expression analysis, a trial was performed to determine what concentration of RNA in the RT reactions would be optimal for analysis. It was determined that by using 100 ng of RNA the expression would be in the linear range of all target genes.

After assuring that the isolated RNA was intact, RT reactions to prepare cDNA from isolated total RNA were performed according to the procedures of Fan et al. (86), using SuperScript III Reverse Transcriptase with the addition of random hexamers. Minus RT reactions (water was used in place of SuperScript III) were used as the negative control. The cDNA was aliquoted into microcentrifuge tubes to decrease the amount of thawing and refreezing, which can damage cDNA.

Expression of Bcl-2, Bax, p21, MCT-1, and Noxa was measured by real-time qRT-PCR using TaqMan Gene Expression Master Mix and Taqman Primers (86). Each

plate was set up with samples from two cell culture runs in duplicate plus a minus RT reaction from each RT run for the gene of interest. A positive control gene (18S) was treated as a separate gene on its own plate due to limited space. The real-time qPCR was completed under standard conditions using the FAM detector on an Applied Biosystems 7900HT Fast Real-Time PCR System for 40 cycles.

Statistical Analyses

All data are presented as means +/- SEM. Cell culture experiments were conducted in triplicate, as a minimum requirement. Gene expression data was transformed from Ct values to expression levels by the following formula: expression = 2^{40-Ct}. Expression values of genes of interest were normalized to 18S expression levels of matching samples. For *in vivo* gene expression, the average Ct value from corresponding 18S samples on all plates was used to calculate 18S expression, which was then used to normalize data. *In vivo* gene expression analysis was completed using Welch's t-test and Shapiro-Wilk normality test in R. Analysis of *in vitro* data was performed using a one way ANOVA, using the generalized linear model in SAS. Differences with $p < 0.05$ were considered significant. Additionally, normality of cell number, proliferation, apoptosis and *in vitro* gene expression data was confirmed by using the D'Agostino-Pearson normality test in GraphPad Prism®.

CHAPTER IV

RESULTS

Experiment I—In Vivo *Results*

Previous work in our lab showed that apigenin and naringenin suppress the preneoplastic lesions of colon cancer by inducing apoptosis and suppressing proliferation in rats (10). Therefore, the goal of the first experiment was to identify how apigenin and naringenin affect proliferation and apoptosis by measuring gene expression of selected genes involved in these two pathways.

Apigenin and Naringenin Alter Gene Expression of Bax, Fas, MCT-1, p21, and TLR-4 but Not Bcl-2 or TLR-2. To determine if apigenin and naringenin altered proliferation and apoptosis through changes in gene expression related to microbial recognition (TLR-2, TLR-4), short chain fatty acid transport (MCT-1), cell cycle (p21), or apoptosis (Bax, Bcl-2, Fas), real-time qRT-PCR was performed on colonic mucosa from rats fed diets of apigenin and naringenin compared to the basal (control) diet. Apigenin suppressed ($p=0.023$) expression of MCT-1, a butyrate transporter, with naringenin leading to an intermediate suppression which approached a significant difference ($p=0.061$) from the basal diet (Figure 5). Additionally, apigenin reduced expression of pro-apoptotic genes Bax ($p=0.05$, Figure 6) and Fas ($p=0.043$, Figure 7). Furthermore, apigenin and naringenin suppressed ($p<0.02$) p21 expression, which is involved with cell cycle arrest (Figure 8). Expression of TLR-4, which is important in

the barrier function of the colon and in mediating inflammatory responses, also was suppressed by apigenin and naringenin ($p<0.01$) (Figure 9). Apigenin and naringenin diets did not significantly alter the expression of Bcl-2 or TLR-2 relative to the basal diet (Table 1).

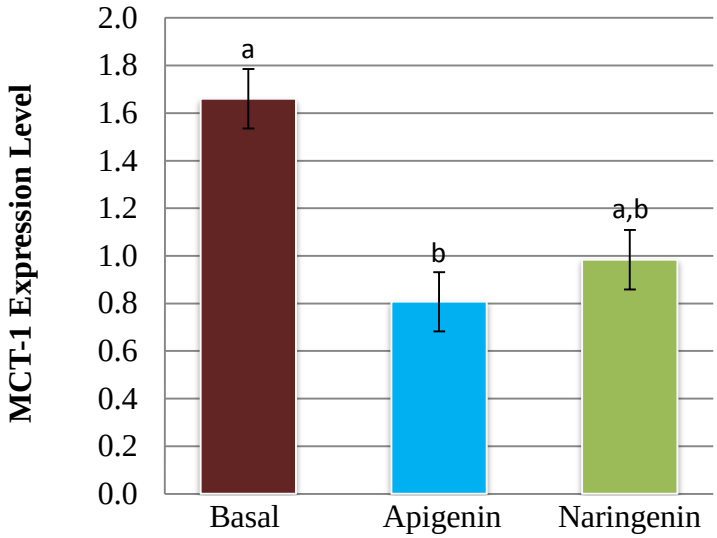

Figure 5. Experimental Diets Decrease MCT-1 Expression. Apigenin suppressed ($p=0.023$) MCT-1 expression (butyrate transporter) compared to basal, whereas naringenin only tended to suppress ($p=0.061$) expression. Values are means +/- SEM, n = 10 rats/group. Expression level normalized with 18S expression.

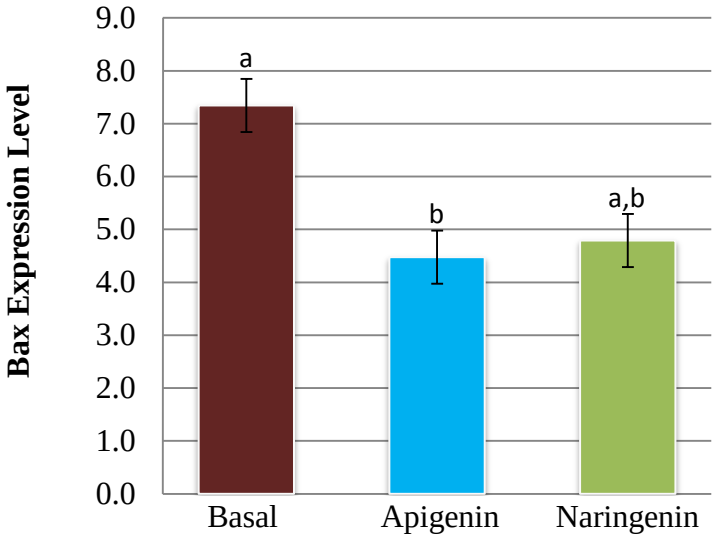

Figure 6. Experimental Diets Decrease Bax Expression. Apigenin suppressed (p=0.05) Bax expression (pro-apoptotic gene) compared to basal, while naringenin tended to suppress (p=0.085) expression Values are means +/- SEM, n = 10 rats/group. Expression level normalized with 18S expression.

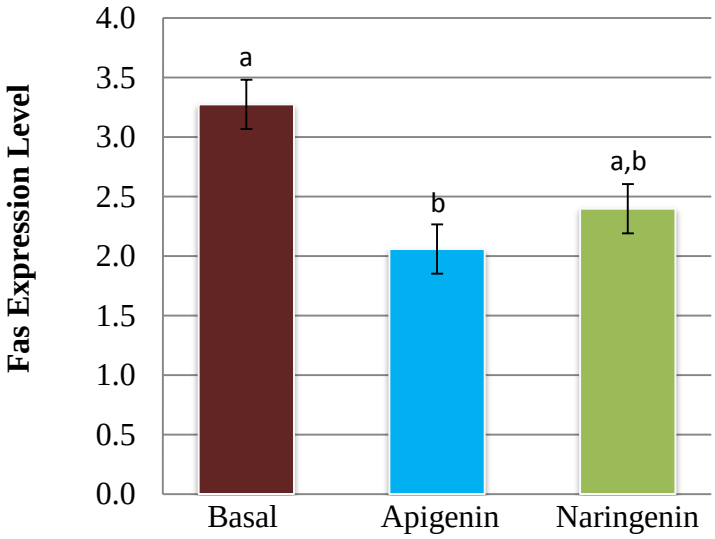

Figure 7. Experimental Diets Decrease Fas Expression. Apigenin suppressed (p=0.043) Fas expression (pro-apoptotic gene) compared to basal, while naringenin tended to suppress (p=0.152) expression. Values are means +/- SEM, n = 10 rats/group. Expression level normalized with 18S expression.

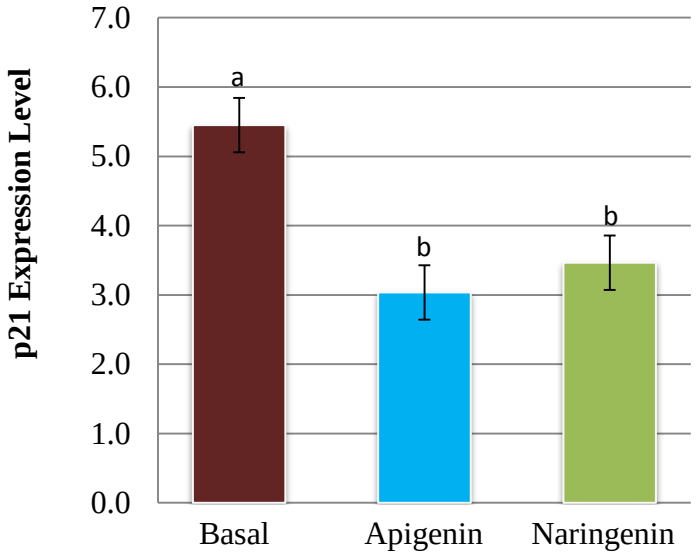

Figure 8. Experimental Diets Decrease p21 Expression. Apigenin and naringenin both suppressed p21 expression (cell cycle regulation) ($p<0.02$). Values are means +/- SEM, n = 10 rats/group. Expression level normalized with 18S expression.

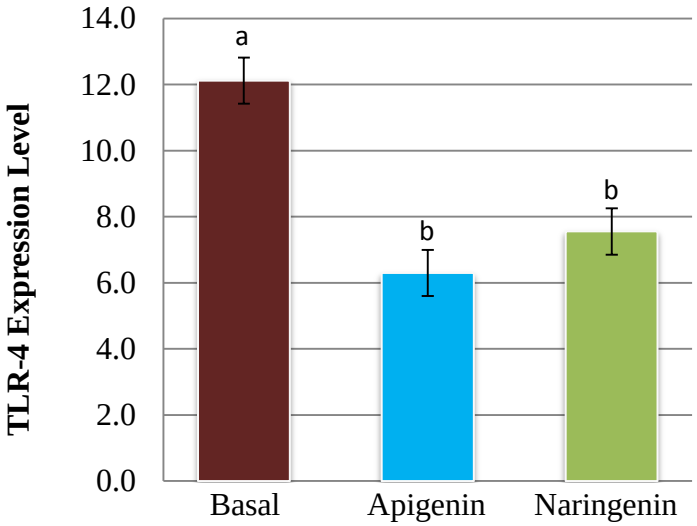

Figure 9. Experimental Diets Decrease TLR-4 Expression. Apigenin and naringenin suppressed TLR-4 expression (microbial recognition and promoter of proliferation) ($p<0.01$). Values are means +/- SEM, n = 10 rats/group. Expression level normalized with 18S expression.

TABLE 1

Effect of Experimental Diets on Expression of Target Genes.[1]

	Target Genes						
Diet Group	Bax	Bcl-2	Fas	MCT-1	p21	TLR-2	TLR-4
Basal	7.344[a]	29.166[a]	3.275[a]	1.660[a]	5.449[a]	1.307[a]	12.120[a]
Apigenin	4.476[b]	23.607[a]	2.059[b]	0.807[b]	3.036[b]	0.948[a]	6.296[b]
Naringenin	4.790[a,b]	21.611[a]	2.398[a,b]	1.138[a,b]	3.464[b]	0.953[a]	7.553[b]
SEM	0.501	1.594	0.208	0.125	0.392	0.079	0.699

[1]Values are given as means +/- SEM, n=10 rats/group. Expression level normalized with 18S expression. Means in a column without a common letter differ significantly ($p<0.05$).

Experiment II*—In Vitro *Results

The results from the *in vivo* gene expression experiment identified some potential mechanisms by which apigenin and naringenin may suppress proliferation and promote apoptosis; however, questions remained, especially whether the chemoprevention induced by apigenin and naringenin was p53-mediated. Therefore, the second part of these investigations was conducted to determine not only the mechanisms involved in chemoprotection induced by apigenin and naringenin using controlled cell culture conditions but also to determine if the effects were p53 dependent. Using non-transformed YAMCs compared to p53 null YAMCs allowed us to investigate both of these aims.

Apigenin and Naringenin Inhibit Non-Transformed Cell Growth but Not Mutated p53 Cell Growth. We measured the effects of apigenin and naringenin treatment on cell growth in non-transformed and mutated p53 YAMCs at non-permissive conditions (37°C). YAMC cells treated with 1 μM or greater apigenin or naringenin exhibited dose-dependent decreases ($p<0.005$) in cell numbers compared to cells treated with the vehicle (DMSO) (Figure 10A). No differences in cell number were identified in the mp53 YAMC cells, except with the highest concentration of apigenin and naringenin ($p< 0.0001$) (Figure 10B). These data demonstrate an inhibitory effect on cell growth in non-transformed cells but not p53 null cells except with a higher concentration. This signifies that whatever mechanisms are involved in reducing cell number with lower concentrations of apigenin and naringenin are in part p53-mediated.

Apigenin and Naringenin Do Not Suppress Proliferation in Non-Transformed or Mutated p53 Colonocytes. To determine the mechanism by which apigenin and naringenin inhibit cell numbers, we first determined their impact on proliferation in non-permissive conditions (39°C). No differences in proliferation were observed with apigenin or naringenin treatment in either cell line compared to the vehicle, except with 20 μM apigenin ($p< 0.0001$) (Figure 11), indicating suppression of cell number in the non-transformed YAMCs is not due to a reduction in proliferation.

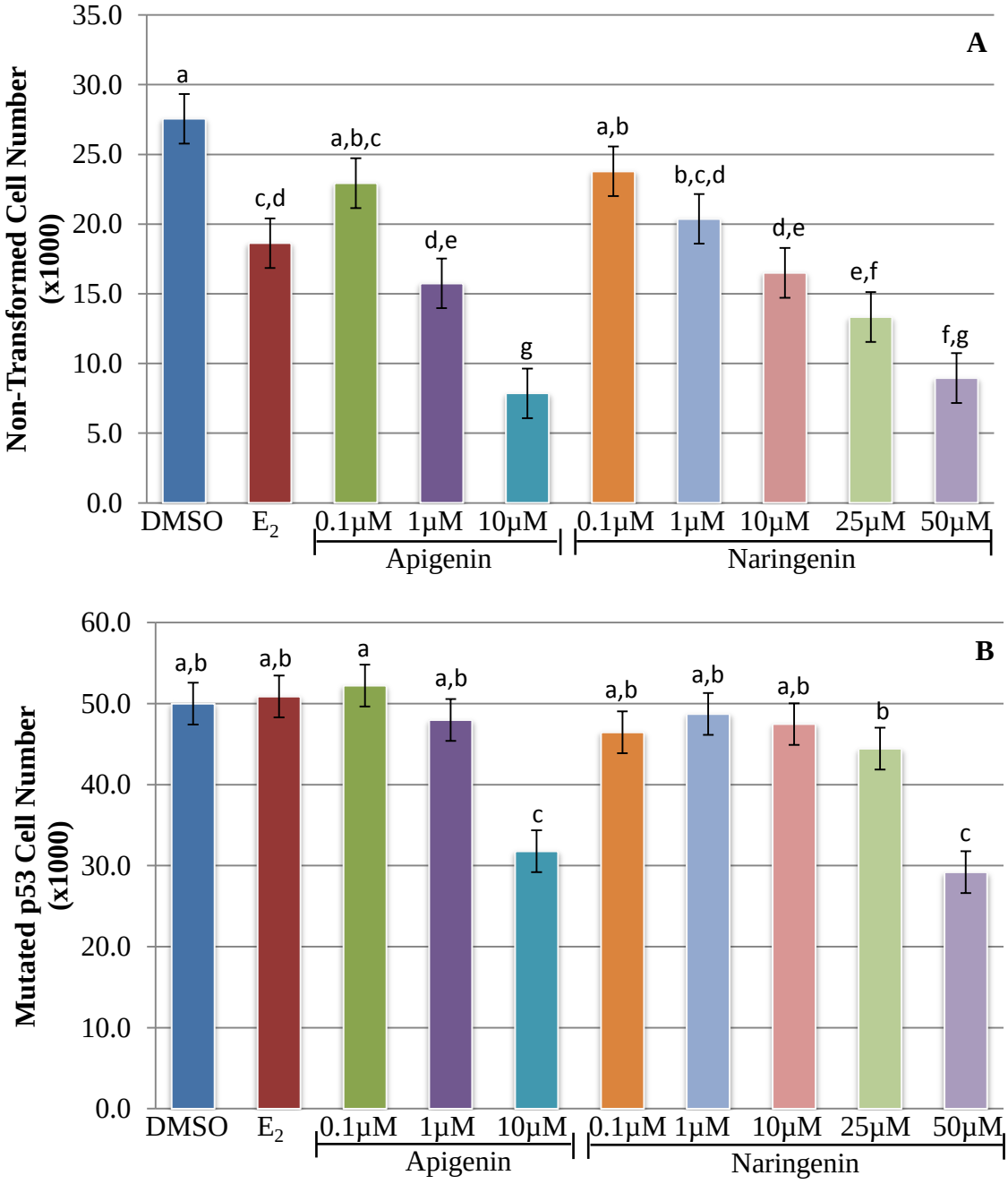

Figure 10. Experimental Treatments Decrease Cell Number in Non-Transformed but Not Mutated p53 YAMCs. A) Non-transformed YAMCs treated with 1 µM or greater apigenin or naringenin exhibited dose-dependent decreases ($p<0.005$) in cell numbers compared to DMSO. B) The only significant differences seen between treatment groups compared to the vehicle treated mp53 YAMCs were with 10 µM apigenin and 50 µM naringenin treatments ($p<0.0001$). Values are means +/- SEM, n = 15 wells/group (5 runs with 3 replicates each).

Apigenin and Naringenin Do Not Induce Apoptosis in Mutated p53 Colonocytes. After discovering that apigenin and naringenin did not inhibit cell number via an inhibition of proliferation, we examined their effect on apoptosis in non-permissive conditions (39°C). No differences were observed with treatment of apigenin or naringenin in either cell line compared to vehicle, even with estradiol treatment (Figure 12B). We have attempted (with multiple runs per attempt) to collect the apoptosis data for the bleo/neo YAMCs. In three of those attempts, there was a high rate of variability within treatment groups per run and inconsistent data between those runs; the attempt with the least variability is shown in Figure 12A. In addition to those problems, there was a lack of response in the positive control for all attempts, raising questions of the validity of the caspase data in the non-transformed YAMCs.

Apigenin and Naringenin Affect Gene Expression of p21, MCT-1, or Noxa in Colonocytes. Even though the reduced cell number in the non-transformed YAMCs was not associated with suppressed proliferation or induction in apoptosis, we were still interested in exploring if any changes in gene expression were occurring with apigenin or naringenin treatment. In the non-transformed YAMCs, no significant differences were seen between any of the treatment groups for Bax, Bcl-2, Noxa, or MCT-1 expression (Table 2). We did note an increased expression of p21 with 1 µM naringenin treatment compared to DMSO, estradiol, and 0.1 and 1 µM apigenin treatments, while expression in cells treated with 10 µM naringenin trended up as well.

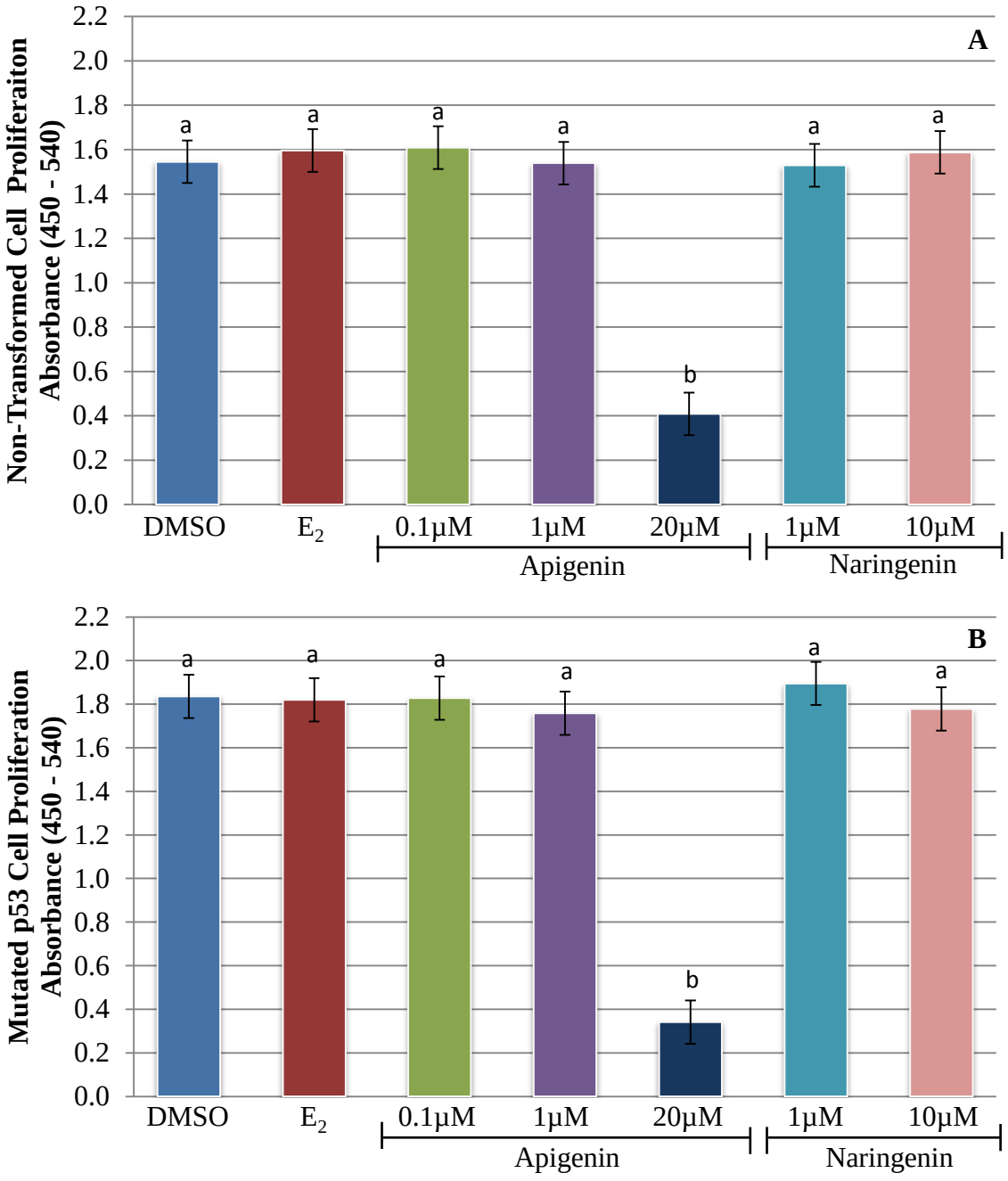

Figure 11. Experimental Treatments Do Not Alter Proliferation. No significant differences in proliferation were observed with treatments of apigenin or naringenin in the non-transformed (A) or mutated p53 (B) YAMCs, except with the 20 µM apigenin treatment ($p < 0.0001$). Values are means +/- SEM, n = 9 wells/group (3 runs with 3 replicates each).

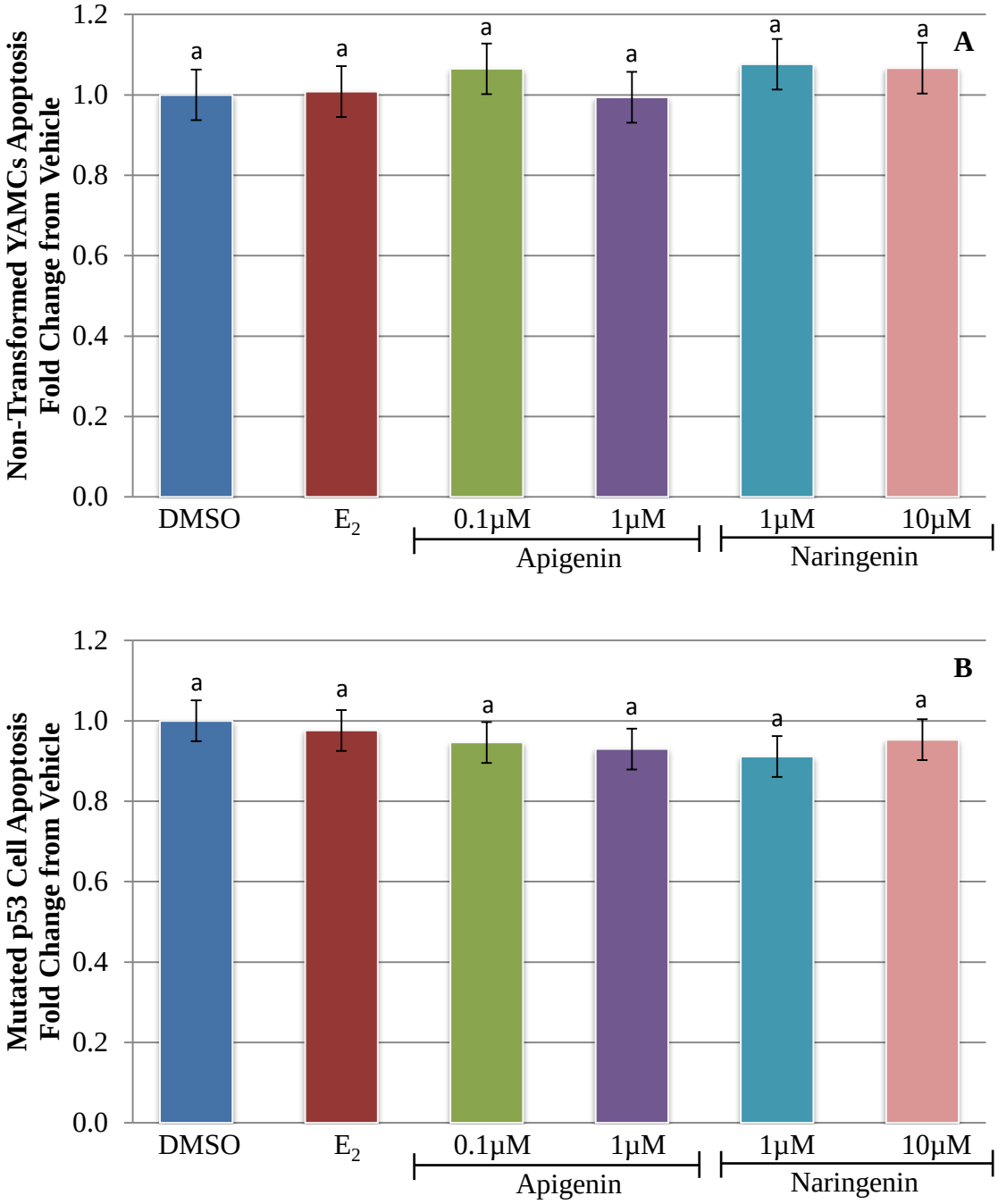

Figure 12. Experimental Treatments Do Not Alter Apoptosis. No significant differences in apoptosis were observed with treatments of apigenin or naringenin in the non-transformed (A) or mutated p53 (B) YAMCs. Values are means expressed as fold change compared to vehicle +/- SEM. For non-transformed YAMCs, n=15 wells/group, (5 runs with 3 replicates each). For mp53 YAMCs, n = 9 wells/group (3 runs with 3 replicates each).

For the mutated p53 YAMCs, there were outliers which correlated with different days on which RT reactions were performed. To preclude those data points from preventing us from identifying possible differences within the data, analysis removing those data points was used to determine significant differences. There were no significant differences seen in the expression of Bax, Bcl-2, or p21 between any of the treatment groups (Table 3), indicating that the reductions in cell number were not mediated through a p53 pathway involving these genes. MCT-1 expression was decreased with 1 µM naringenin compared to DMSO and 10 µM naringenin. Additionally, 1 µM naringenin also increased the expression of Noxa compared to 1 µM apigenin. Treatment of 10 µM naringenin also elicited an increase in Noxa expression compared to DMSO, E_2, and 1 µM Apigenin. This indicates one possible mechanism for how the 50 µM naringenin treatment reduced cell number in the cell growth assay is via an increase in expression of Noxa, a pro-apoptotic gene.

TABLE 2
Effect of Experimental Treatments on Expression of Target Genes in Non-Transformed YAMCs.[1]

	Target Genes				
Treatments	Bax	Bcl-2	Noxa	MCT-1	p21
DMSO	10.658[a]	12.567[a]	5.679[a]	18.533[a]	20.976[a]
E_2	10.572[a]	13.059[a]	5.479[a]	18.472[a]	19.410[a]
0.1 µM Apigenin	10.436[a]	13.246[a]	5.470[a]	17.249[a]	19.561[a]
1 µM Apigenin	10.439[a]	13.531[a]	6.011[a]	18.282[a]	21.027[a]
1 µM Naringenin	12.437[a]	16.488[a]	6.150[a]	21.881[a]	26.683[b]
10 µM Naringenin	11.083[a]	14.024[a]	5.762[a]	17.880[a]	22.556[a,b]
SEM	1.450	2.406	0.579	2.671	1.987

[1]Values are given as means +/- SEM, n=9 wells/group. Expression level normalized with 18S expression. Means in a column without a common letter differ significantly ($p<0.05$).

TABLE 3

Effect of Experimental Treatments on Expression of Target Genes in Mutated p53 YAMCs.[1]

	Target Genes				
Treatments	Bax	Bcl-2	Noxa	MCT-1	p21
DMSO	7.550[a]	19.283[a]	2.915[a,b]	29.519[a]	17.758[a]
E_2	6.978[a]	20.569[a]	2.892[a,b]	26.365[a,b]	16.027[a]
0.1 µM Apigenin	6.729[a]	21.313[a]	3.103[a,b,c]	25.189[a,b]	17.044[a]
1 µM Apigenin	7.084[a]	16.585[a]	2.820[a]	24.724[a,b]	16.453[a]
1 µM Naringenin	7.363[a]	20.237[a]	3.262[b,c]	22.024[b]	16.499[a]
10 µM Naringenin	7.932[a]	18.331[a]	3.377[c]	28.665[a]	18.001[a]
SEM	0.492	1.858	0.159	2.313	0.948

[1]Values are given as means +/- SEM, n=9 wells/group; however, a few outliers were removed. Expression level normalized with 18S expression. Means in a column without a common letter differ significantly ($p<0.05$).

CHAPTER V

DISCUSSION

The work described in this thesis was a two part study. The purpose of the first part of these experiments was to investigate the mechanisms involved in the reduced high multiplicity aberrant crypt foci, HMACF, reduced proliferation, and increased apoptosis seen in a previous *in vivo* study in our laboratory (10). Using real-time qRT-PCR, it was determined that apigenin and naringenin were able to reduce the expression of Bax and Noxa (apoptosis), MCT-1 (short chain fatty acid transport), p21 (cell cycle regulation), and TLR-4 (microbial recognition). No change was seen in the expression of Bcl-2 or TLR-2.

Several studies have reported an anti-proliferative effect with treatment of apigenin and naringenin and have related this to a reduction in p21 protein expression (52-53, 60-61, 89). Although there was a reduction in proliferation seen with animals fed apigenin or naringenin diets compared to the basal diet, this change contradicts the expected increase in p21 expression. We believe that the decrease in p21 expression is a result of the reduction in MCT-1 expression. Though, there is not research supporting a direct link to MCT-1 activity and expression to p21 expression, butyrate has been shown to upregulate p21 expression (30). Additionally, Hu et al. showed that butyrate treatment decreased HCT-116 (colon cancer cells) proliferation, which was associated with an increase in both p21 protein and mRNA expression (90). As mentioned in the literature review, butyrate has the potential to reduce tumorigenesis via epigenetic modulations

(26, 32). Considering that there was a protection against preneoplastic lesions, the reduction in MCT-1 expression is also opposite of what we expected. There is, however, evidence that supports polyphenols interfering with butyrate's chemoprotective abilities. One study showed that green tea phenolic compounds impaired butyrate's entry into the cell via interruption of the MCT-1 localization within lipid rafts (91). Conversely, another study demonstrated that a butyrate-rich colonic microenvironment may select for cancer cells that can metabolize butyrate, which tend to be more aggressive (92). Though we did not relate the increased apoptosis and reduced proliferation seen in animals fed apigenin or naringenin to an increase in p21, we did discover that these compounds may be interfering with the butyrate availability within the colonocytes via a reduction in MCT-1 expression, thus reducing p21 expression instead. Perhaps, the reduction in MCT-1 expression with apigenin and naringenin may be beneficial depending on whether the environment has selected for the more aggressive tumor cells that are able to utilize butyrate as an energy source.

On the other hand, changes in *in vivo* TLR-4 expression are consistent with our hypothesis that apigenin and naringenin's chemoprotection may be due to transcriptional regulation of this gene. Dou et al. (93) revealed that naringenin was able to reduce the severity of inflammation in dextran sulfate sodium (DSS)-induced murine colitis, resulting in downregulation of pro-inflammatory mediators. These changes where associated with lower TLR-4 protein and mRNA expressions and lower phospho-NF-κB p65 protein expression (93). Additionally, apigenin and genistein have both been shown to have anti-inflammatory effects via suppression of TLR-4 and NF-κB activity in LPS-

induced models, reducing pro-inflammatory cytokine production (94-95). Specifically, apigenin was able to suppress p65 phosphorylation (95). With this understanding, it appears that apigenin and naringenin may be chemoprotective due to reduction in TLR-4 expression and inhibition of the TLR-4/ NF-κB pathway.

It is interesting that the expressions of the selected pro-apoptotic genes were reduced by apigenin and that the anti-apoptotic gene was unaffected by both flavonoids. This is contradictory to literature that shows increased pro-apoptotic gene or protein expression and reduced anti-apoptotic gene and protein expression (52-54). One possibility for these unexpected results is that, since the apoptosis was only seen at the luminal surface, the proportion of apoptotic cells on the luminal surface to the non-apoptotic cells within the crypt washed out any possible detectable effect. Additionally, other cells, most likely from the lamina propria, may have also been collected during scraping. Furthermore, it may indicate that other genes upstream or downstream within the apoptotic pathways are transcriptionally regulated instead to increase apoptosis. For example, apigenin and naringenin have been demonstrated to modify p53 protein and gene expression as well as activate the MAPK/ERK 1/2 pathway (51-52, 54-57, 60). Though we did base our gene selection on research suggesting possible regulation via those genes, utilizing an apoptosis PCR array may have been a more constructive approach to understanding how apigenin and naringenin increased apoptosis. Additionally, since there were unexpected changes seen in the transcriptional regulation of several target genes, it would be illuminating to measure protein expression of

enzymes related to apoptosis and cell cycle arrest to determine possible translational regulation.

The estimated concentration of apigenin and naringenin that decreased the number of preneoplastic lesions *in vivo* varied from the concentration that reduced cell number *in vivo*. This may be due to *in vivo* interactions between the microbiota and the flavonoids of interest. Probable changes in the colon microbiota by apigenin and naringenin are reflected by changes in the microbial pattern recognition receptor TLR-4 and the butyrate transporter MCT-1. Polyphenols have been reported to modify the gut microbiome (69, 96). In one study, polyphenols including chlorogenic acid, caffeic acid, and quercetin were shown to stimulate *Bifidobacteria* and decrease the ratio of *Firmicutes* to *Bacteriodes* (96). Conversely, apigenin and naringenin are also able to be biotransformed by gut bacteria. Rechner et al. (97) showed that naringin and naringenin along with other polyphenols have multiple degradation products. That degradation can depend on flavonoid concentration, individual microflora composition, and specific flavonoid structural differences (97). Furthermore, gut microbial metabolism of polyphenols from black tea and red wine or grape juice was demonstrated to be source and colon region specific (98). Shifts in the colon microbiome as well as modifications (increasing bioavailability) or metabolism (producing additional bioactive metabolites) of apigenin and naringenin by colonic bacteria may explain the differences in concentration sensitivity between the *in vivo* and *in vitro* studies.

The second part of these investigations was to determine not only the mechanisms involved in chemoprotection induced by apigenin and naringenin using

controlled cell culture conditions but also to determine if the effects were p53 dependent. Additionally, it was of interest to compare changes to those resulting from estradiol treatment in order to identify if they have similar effects.

In vitro, it was determined that a dose dependent decrease in cell number occurred with apigenin and naringenin treatment in the non-transformed YAMCs but not in their counterparts, the mutated p53 YAMCs, except at the highest concentration tested. These data substantiate the conclusions from the previous *in vivo* study that apigenin and naringenin may protect against colon cancer (10). Additionally, it is clear that the dose-dependent reduction in cell number by apigenin and naringenin is at least in part p53-mediated, especially at lower concentrations. This is consistent with other research that indicates that apigenin and naringenin can stimulate the p53 response pathway (51-53, 60). It was also determined that concentrations of 0.1 and 1 μM apigenin and 1 and 10 μM naringenin elicit an effect similar to estradiol, which has proven to reduce the number of preneoplastic lesions (77). The reduction of cell numbers in mp53 YAMCs resulting from the greatest concentrations of apigenin and naringenin treatments suggests alternate pathways can be induced by these flavonoids with a high threshold value. Gene expression analysis indicated that naringenin was able to induce expression of Noxa, a pro-apoptotic gene, compared to vehicle treated mp53 YAMCs.

The effect on cell number is not linked directly to suppression of colonocyte proliferation in either p53 competent or mutant colonocytes. This does not support the reduction in proliferation documented *in vivo* (10), indicating that changes in proliferation may only be seen after exposure to a carcinogen. However, it does

correspond with previous studies where estradiol treatment in bleo/neo and mp53 YAMCs do not reduce proliferation (76, 80). Moreover, treatment of 1 µM naringenin did significantly increase expression of p21 in the non-transformed YAMCs, with 10 µM naringenin treatment trending up as well. This effect was not seen in the p53 null YAMCs. This may indicate that naringenin, at a higher concentration, may induce cell cycle arrest.

Furthermore, there was no significant change in apoptosis with treatment of apigenin or naringenin in either non-transformed or p53 null YAMCs. The lack of change in the mutated p53 YAMCs is consistent with previous estradiol treatment of these cells (76, 80) as well as with literature indicating that these flavonoids act via a p53-mediated pathway (51-53, 60). However in the non-transformed YAMCs, apoptosis measurements were inconclusive due to the lack of induction with the positive control (estradiol). There were multiple attempts to collect the apoptosis data in the bleo/neo YAMCs, including an alternate assay. In most of those attempts, there was a high rate of variability within treatment groups per run and inconsistent data between those runs. The issues could not be resolved within the timeframe of this thesis; however, several factors may be involved. One concern was the change in non-permissive temperatures between the cell number assays and the other *in vitro* experiments. There is conflicting literature on what temperature is most appropriate as a non-permissive condition, ranging from 37° to 39.5°C (76, 99-102). The temperature change was made due to evidence indicating that 39°C was the more appropriate non-permissive condition for the bleo/neo YAMCs, although a collaborator's lab had used 37°C in the past (76). A change in

temperature could possibly have caused a loss in sensitivity and therefore a diminishment in the effect expected, but this is unlikely due to evidence showing reduced apoptosis via estradiol treatment at both temperatures (76, 80). Another issue is that these cells are highly sensitive based on reports from the donor. One example of this is that the non-transformed YAMCs are unable to be transported on dry ice. It may have been beneficial to use a less sensitive YAMC line with a corresponding p53 null cell line. Yang et al. (82) demonstrated an induction in apoptosis in less primary YAMCs with sorghum phenolic compounds and estradiol. However, the unique opportunity of using a primary cell to determine chemoprotective abilities would be lost. Another limitation is the fact that the cells are treated for 96 hours with media changed after 48 hours. This could select for the more hardy cells that may not be sensitive or affected by the various treatments, similar to the idea that a butyrate-rich environment may select for more aggressive tumor cells (92). Though in the past it has not been necessary to normalize data to cell number in order to identify an induction in apoptosis with estradiol treatment, making this adjustment would provide a better apoptotic index.

Similar to the apoptosis assays, the *in vitro* gene expression data was also inconclusive due to the lack of response of the positive control. Estradiol has been shown to induce expression of Bax and Noxa while reducing expression of Bcl-2 in bleo/neo YAMCs (76). As mentioned earlier, there was an increase in Noxa expression with naringenin treatments in the p53 null YAMCs. This suggests that one possible mechanism of how 50 µM naringenin treatment reduced cell number in mp53 YAMCs is via an increase in Noxa expression. Additionally, the fact that 1 µM naringenin

treatment led to a reduction in MCT-1 expression in the mp53 YAMCs confirms the change seen *in vivo*. Further investigation would be valuable to reassess the apoptosis induction and various gene expressions in non-transformed YAMCs treated with apigenin and naringenin.

Overall the purpose of these experiments was to determine how apigenin and naringenin may decrease risk of colon carcinogenesis, specifically in the early stages, by first determining the mechanisms of apigenin and naringenin *in vivo* and then by further testing our hypothesis *in vitro*.

In vivo, the increased apoptosis and reduced proliferation with apigenin and naringenin diets seen in a previous *in vivo* experiment were not related to an increase in p21 expression. However, we did discover that these flavonoids may interfere with the butyrate availability within the colonocytes, thus reducing p21 expression instead. This reduction in MCT-1 expression may help prevent against selection for the more aggressive tumor cells that are able to utilize butyrate as an energy source. Additionally, the reduced expression of TLR-4 suggests that apigenin and naringenin are able to modulate the inflammatory response, which promotes carcinogenesis, via the MyD88 pathway that ultimately activates NF-κB. These changes may have contributed to apoptosis promotion and proliferation suppression to prevent preneoplastic lesions.

In vitro, the dose-dependent reduction in cell number by apigenin and naringenin treatment in non-transformed but not p53 null YAMCs supports the previous *in vivo* conclusion that these flavonoids may be chemoprotective. This also indicates that the mechanism of action is at least in part p53-mediated. However, unlike the *in vivo* study,

the reduced cell number was unrelated to a reduction in proliferation. Additionally, the apoptosis and gene expression data were inconclusive for the non-transformed YAMCs. For the mutated p53 YAMCs, there was an increase in Noxa with naringenin treatment but not a corresponding induction in apoptosis. Additionally, 1 μM naringenin significantly reduced expression of MCT-1, supporting the *in vivo* analyses. Though more research is necessary to determine the specific mechanisms that reduced the cell number with apigenin and naringenin treatment, it appears to be in part p53-mediated.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Previous work in our lab showed that rats injected with AOM had a lower incidence of HMACF, a preneoplastic lesion of the colon, when fed diets containing apigenin and naringenin compared to the basal diet. This chemoprotection was related to an increase in apoptosis at the luminal surface and a reduction in proliferation of colonocytes. The work in this thesis continued exploring the mechanisms involved in this protection. We found that apigenin and naringenin treatment of these animals led to a decrease in the gene expressions of Bax, Fas, MCT-1, p21, and TLR-4. There, however, was no significant change in Bcl-2 or TLR-2 gene expression. Suppressed expression of Bax and Fas by apigenin was contradictory to expected results. Since the increased apoptosis in the previous *in vivo* study was only seen at the luminal surface, we believe that the ratio of apoptotic cells from the luminal surface to the non-apoptotic cells within the crypt may have washed out any detectable increase in the expression of these genes. Additionally, other genes may be involved that we did not measure. The reduced proliferation with apigenin and naringenin diets seen in the previous *in vivo* study was not related to an increase in p21 expression. Yet, the discovery that these flavonoids may interfere with the butyrate availability within colonocytes via a reduction in MCT-1 expression explains why p21 expression is reduced instead. Additionally, suppression of TLR-4 expression alludes to the ability that apigenin and naringenin can downregulate the inflammatory response related to the MyD88/NF-κB pathway. These

data suggest that changes in transcriptional regulation are able to reduce the risk of carcinogenesis.

In vitro, we discovered a dose dependent decrease in cell number of non-transformed YAMCs with treatment of apigenin and naringenin but not in YAMCs with a mutated p53. Further work showed that this cell number reduction was not related to changes in proliferation. We attempted to connect the cell number decrease with apoptosis and related gene expression; however, the data was inconclusive and unreliable due to a lack of effect in the positive control. We believe that a possible cause is poor cell state related to the highly sensitive nature of primary cells. Due to the dose-dependent reduction in non-transformed cell number but not mutated p53 cell number, we did conclude that the effects of apigenin and naringenin are in part p53-mediated. The reduction of cell numbers in mp53 YAMC cells resulting from the greatest concentrations suggests alternate pathways can be induced by these flavonoids at higher concentrations. Further investigation would be valuable to reassess the apoptosis induction and various gene expressions in YAMCs treated with apigenin and naringenin.

Research involving bioactive compounds is a field that deserves continued attention especially considering the research that links compounds such as apigenin and naringenin to health promotion and disease prevention. Specifically, the data collected for this thesis add to the understanding of mechanisms involved in apigenin and naringenin's chemoprotective ability. More research will need to be completed to better appreciate exactly how apigenin and naringenin impact biochemical pathways.

In addition to better understanding how these flavonoids impact a particular disease state, further examination of apigenin and naringenin should also be focused in areas of bioavailability, availability from dietary sources, dietary intake, biochemical pathway involvement, timing of intake for protection, and possible interactions with current disease treatments. With a larger accumulation of data in all areas related to apigenin and naringenin, we hope that someday there will be dietary recommendations for these flavonoids as well as other bioactive compounds.

NOMENCLATURE

18S	18S ribosomal RNA
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOM	Azoxymethane
Bax	Bcl-2-like protein 4
Bcl-2	B-cell lymphoma 2
BrdU	Bromodeoxyuridine
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CO_2	Carbon dioxide
Cox-2	Cyclooxygenase-2 or Prostaglandin-endoperoxide synthase 2
Ct	Cycle threshold
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSS	Dextran sulfate sodium
E_2	Estradiol
ERK 1/2	Extracellular-signal-regulated kinase

ERα/β	Estrogen receptor α or β
FAM	6-carboxyfluorescein
Fas	TNF receptor superfamily, member 6
FasL	Fas ligand or cluster of differentiation 95 ligand (CD95L)
FBS	Fetal bovine serum
HDAC	Histone deacetylase
HMACF	High multiplicity crypt foci
HRT	Hormone replacement therapy
IFN-γ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases
MCT-1	Monocarboxylate transporter 1 (SLC16A1)
MMP-9	Matrix metalloproteinase-9
mp53	Mutated p53
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1

p21WAF1/CIP-1	Cyclin-dependent kinase inhibitor 1
p53	Tumor suppressor p53
p65	Transcription factor p65 or NF-κB p65 subunit
pCMBS	*p*-chloromercuribenzene sulfonate
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcription
SAS	Statistical Analysis System
SEM	Standard error of the mean
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
TRIF	TIR-domain-containing adapter-inducing interferon-β
US	United States
YAMC	Young adult mouse colonocytes

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